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Title: Delivery of β-carotene to the in vitro intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifiers

Article Type: Research paper

Keywords: β-carotene; nanoemulsions; in vitro digestion; intestinal barrier

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Abstract: To increase the intestinal delivery of dietary β-carotene, there is a need to develop nanostructured food systems to encapsulate this fat soluble bioactive. The aim of this study was to evaluate the bioacessibility and bioavailability across the intestinal barrier of β-carotene-enriched nanoemulsions stabilised with two emulsifiers (lecithin or sodium caseinate) by coupling an in vitro gastrointestinal digestion with two in vitro cell culture models (Caco-2 or co-culture of Caco-2/HT29-MTX). Nanoemulsions stabilised with lecithin had significantly higher β-carotene in the gastrointestinal digested micellar fraction, lower β-carotene in the Caco-2 (and Caco-2/HT29-MTX) apical compartment and significantly higher β-carotene in Caco-2 cellular content compared to β-carotene-enriched nanoemulsions stabilised with sodium caseinate. Finally, to assess anti-inflammatory activity of digested nanoemulsions, lipopolysaccharide stimulated macrophages were exposed to Caco- 2 basolateral samples with levels of TNF-α and IL-β, subsequently quantified. A TNF-α response from stimulated THP-1 macrophages was elicited by basolateral samples, regardless the emulsifier used to formulate nanoemulsions. This study demonstrated that β-carotene permeability is influenced by the food derived emulsifier used for stabilising nanoemulsions, indicating that composition may be a critical factor for β-carotene delivery

Linda Giblin,
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14th May 2020

Dear Editor,

Please find attached the revised version of the manuscript entitled "Delivery of β-carotene to the in vitro intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifiers". Firstly, we would like to thank the editor and the reviewers for their time and constructive comments. We have carefully addressed all the comments. The corresponding changes made in the revised paper are summarized below and corrections have been highlighted in red along the manuscript to facilitate further revaluation.

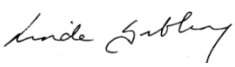
We hope that after this revision, you will now find our manuscript suitable for publication in LWT-Food Science and Technology journal.

Thank you for consideration.

Looking forward to hearing from you.

Yours sincerely,

Linda Giblin on the behalf of the authors



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Answer to Reviewers' comments

Please find enclosed the revised version of the manuscript *Delivery of β-carotene to the in vitro intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifiers*.
We would like to thank the reviewers for careful and thorough reading of this manuscript and for the thoughtful comments and constructive suggestions, which help to improve the quality of this manuscript. Corrections and changes have been highlighted in red along the text to facilitate further revaluation.

Reviewers' comments:

Reviewer #1:
This is an interesting in vitro evaluation of the b-carotene intestinal delivery, mainly for the planned approach regarding all cell lines studies. However, these are some concerns you may correct or explain
1) Looking at previous published recent papers by some of the same authors, I found a few with similar approach to this one, including from some of same listed authors.
DOI: 10.1039/C8FO02069H
DOI: 10.3390/foods9040447
DOI: 10.3390/foods9030325
2) Therefore, I wonder why you excluded them, instead of discussing them with these recent findings.
3) In that case, the originality of this manuscript gets compromised.
We understand reviewer's concern and we have included/discussed these recent publications in the manuscript (lines 63-67, 85-87, 221 and 237-240). Recently, we and others have shown the release of β-carotene encapsulated in nanoemulsions after *in vitro* gastrointestinal digestion; however the originality of this study focuses in the *in vitro* permeability of β-carotene across the intestinal barrier, using a 21 days differentiated co-culture of Caco-2/HT29-MTX cells model. Other authors had solely looked at absorption of encapsulated β-carotene in undifferentiated adherent Caco-2 cells (Lu, Kelly, & Miao 2017) which do not have the absorptive and secretive enteric phenotypes of an intestinal barrier. This is stated in lines 71-73: "Lu, Kelly, & Miao (2017) described that the permeability of β-carotene in undifferentiated naïve gastrointestinal epithelial cells". We also state in lines 74-76 the reason why you must use differentiated monolayers for permeability studies: "To model absorption *in vitro*, the use of differentiated monolayers expressing tight junctions, best represent the morpho-functional features of the intestinal barrier (Guri, Gülseren, & Corredig, 2013). In addition we use HT29-MTX cells, these add a layer of complexity to the *in vitro* barrier as they are mucus producers and therefore their inclusion brings *in vitro* model data closer to real life.
In our previous publication, Gasa-Falcon et al. (2019), we focused on the evaluation of physicochemical properties of β-carotene enriched nanoemulsions and, apart from particle size and <zeta>-potential, we also used optical microscopy to study the microstructure of β-carotene enriched nanoemulsions. Moreover, we determined the particle size and <zeta>-potential along the *in vitro* gastro intestinal transit. In the present study, particle size and <zeta>-potential are included to provide evidences of adequate nanoemulsions preparation, as the aim of the study was then to build on previous work by evaluating the permeability of β-carotene-enriched nanoemulsions across 21 days differentiated intestinal barrier models *in vitro*, after *in vitro* static GID.

4) Author should correct RDAs from FDA and EFSA. Both should be expressed in RAE. In fact, they are very similar, opposite what is written in line 58. The RDA-FDA for Vitamin A is 900 ug RAE, while the PRI-EFSA is 750 ug RAE.
We apologise if we did not include the correct RDAs in RAE. Values were corrected and included in lines 56-58: "the U.S. Food and Drug Administration and the European Food Safety Authority derive a RDA for vitamin A of 900-700 µg of retinol activity equivalents (RAE) daily and a population reference intake of 750-650 µg REA daily, respectively."

Reviewer #3:
The authors hoped to explore the effect of emulsifiers type (lecithin or sodium caseinate) on the delivery of <beta>-carotene to the in vitro intestinal barrier using nanoemulsions but partly similarly with previous own research. However, there are some researches about the stability of beta-carotene nano emulsions stabilized by caseinate (The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions, Food Hydrocolloids, 2014, 35: 19-27), and even including your own accepted manuscript (Title: Impact of emulsifier nature and concentration on the stability of <beta>-carotene enriched nanoemulsions during in vitro digestion) published in Food & Function at 2019. So, what are the differences or highlights between your present manuscript and other researches? Unfortunately, the manuscript cannot be accepted this moment before fulfilling the requirements due to other experimental design questions.
We agree with the reviewer. We have now included the differences in lines 63-67 and 81-83. In summary, previous studies have determined the release of β-carotene encapsulated in nanoemulsions along the gastrointestinal tract (Yi et al., 2014; Gasa-Falcon et al., 2019) however, to the best of our knowledge, this is the first time that its permeability *in vitro,* using 21 days differentiated mature intestinal barrier models (Caco-2/HT29-MTX co-cultures), is assessed. Other authors have solely looked at absorption in undifferentiated adherent Caco-2 cells (Lu, Kelly, & Miao 2017) which do not have the absorptive and secretive enteric phenotypes of an intestinal barrier. This is stated in lines 71-73: "Lu, Kelly, & Miao (2017) described that the permeability of β-carotene in undifferentiated naïve gastrointestinal epithelial cells". We also state in lines 74-76 the reason why you must use differentiated monolayers for permeability studies: "To model absorption *in vitro*, the use of differentiated monolayers expressing tight junctions, best represent the morpho-functional features of the intestinal barrier (Guri, Gülseren, & Corredig, 2013)". In addition we use HT29-MTX cells, these add a layer of complexity to the *in vitro* barrier as they are mucus producers and therefore their inclusion brings *in vitro* model data closer to real life.

(1) The number of experimental samples is not enough to reflect the experiment designs well.
We apologise for this omission in the text. In reality experiments were performed in triplicate on at least two experimental days and data was expressed as the mean with standard deviation. This is now stated in line 212.

(2) It's not enough to show the physicochemical properties of nanoemulsions only characterized by particle size, <zeta>-potential, and instability index? Which is different from the above research published in Food & Function in 2019.
We thank the reviewer for this comment. In our previous publication, Gasa-Falcon et al. (2019), we focused on the evaluation of physicochemical properties of β-carotene enriched nanoemulsions and, apart from particle size and <zeta>-potential, we also used optical microscopy to study the microstructure of β-carotene enriched nanoemulsions. Moreover, we

determined the particle size and <zeta>-potential along the *in vitro* gastro intestinal transit. In the present study, particle size and <zeta>-potential are included to provide evidences of adequate nanoemulsions preparation, as the aim of the study was then to evaluate the permeability of β-carotene-enriched nanoemulsions across 21 days differentiated intestinal barrier models *in vitro*, after *in vitro* static GID. To the best of our knowledge, this is the first time that the permeability of β-carotene-enriched nanoemulsions is reported using a 21 days differentiated model of the intestinal barrier using Caco-2/HT29-MTX co-cultures. Gasa-Falcon et al. (2019) and related studies are now referred in lines 63-67, 85-87, 221 and 237-240.

(3) The droplet size and <zeta>-potential should be added after static simulated digestion.
We agree with the reviewer as we have previously published a fully characterization of droplet size and <zeta>-potential during and after static simulated digestion of β-carotene nanoemulsions stabilised with lecithin and sodium caseinate (Gasa-Falcon et al., 2019). We have now referred to our previous results in lines 237-240: "Interestingly, ζ-potential of lecithin stabilised β-carotene nanoemulsions became less negative after in vitro GID, while NaCas emulsions post GID have a stronger negative value (Gasa-Falcon et al., 2019). In that previous study, particle size after in vitro GID in lecithin nanoemulsions was higher compared to NaCas."

(4) Why was the particle size and instability index characterized by both Table 1 and Fig 1?
We agree with the reviewer as the instability index was characterized in Figure 1B and Table 1 (end point instability index) therefore these results have been deleted from Table 1. Regarding particle size, Figure 1A includes particle size distribution of formulated nanoemulsions, which provides information of distribution homogeneity. On the other hand, Table 1 indicates the average of their particle size (surface mean diameter (d_{43} (μm)), calculated from particle size distribution results (Figure 1A). As both parameters provide valuable information we prefer to maintain them.

(5) Maybe it's better to condense the introduction in tables and figures captions.
Following reviewer's suggestion, we have condensed tables and figures captions.

Reviewer #4:
Very good, thorough work and write-up. Very well referenced.
Only minor comments/questions:
--lines 117-118: What was the exact procedure for determining the physical stability of the nanoemulsions using centrifugation?
Physical stability of nanoemulsion was determined using an analytical centrifuge LUMiSizer 6112. A centrifuge force is applied while transmitted light across the sample length is recorded. The procedure has been included in lines 123-125 "Physical stability of β-carotene enriched nanoemulsions was determined with an analytical centrifuge LUMiSizer 6112 (L.U.M. GmbH, Berlin, Germany) that accelerates destabilisation of samples. Results were analysed using the software package SEPView 6.0 (L.U.M. GMBH) that records transmitted light across the sample length and calculates the instability index".

--Figure 1B: "Instability Index" is not defined anywhere.
We agree with the reviewer and we have now included instability index definition in lines 125-126: "instability index that ranges from 0 to 1, with the greatest instability at 1".

REFERENCES

Gasa-Falcon, A.; Odriozola-Serrano, I.; Oms-Oliu, G.; Martín-Belloso, O. Impact of emulsifier nature and concentration on the stability of β-carotene enriched nanoemulsions during *in vitro* digestion. *Food Funct.* 2019, *10*, 713–722.

Lu, W., Kelly, A., & Miao, S. (2017). Bioaccessibility and cellular uptake of β-carotene encapsulated in model o/w emulsions: influence of initial droplet size and emulsifiers. *Nanomaterials*, 7(9), 282.

Salvia-Trujillo, L., Qian, C., Martín-Belloso, O., McClements, D.J. Influence of particle size on lipid digestion and β-carotene bioaccessibility in emulsions and nanoemulsions. *Food Chemistry*, 2013, *141*(2), 1472-80.

Teixé-Roig, J., Oms-Oliu, G., Ballesté-Muñoz, S., Odriozola-Serrano, I., Martín-Belloso, O. Improving the *in vitro* bioaccessibility of β-carotene using pectin added nanoemulsions. *Foods, 9*(4), 447.

Yi, J., Li, Y., Zhong, F., & Yokoyama, W. (2014). The physicochemical stability and in vitro bioaccessibility of beta-carotene in oil-in-water sodium caseinate emulsions. *Food Hydrocolloids, 35*, 19-27.

Highlights

- Permeability of β-carotene was assessed using *in vitro* cell culture models.
- Bioaccessibility of β-carotene was enhanced with lecithin-stabilised nanoemulsions
- Nanoemulsions elicited a TNF-α response from stimulated THP-1 macrophages
- This study elucidates the hurdles faced by concentration limits

Delivery of β-carotene to the *in vitro* intestinal barrier using nanoemulsions with lecithin or sodium caseinate as emulsifiers

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Abstract

To increase the intestinal delivery of dietary β-carotene, there is a need to develop nanostructured food systems to encapsulate this fat soluble bioactive. The aim of this study was to evaluate the bioacessibility and bioavailability across the intestinal barrier of β-carotene-enriched nanoemulsions stabilised with two emulsifiers (lecithin or sodium caseinate) by coupling an *in vitro* gastrointestinal digestion with two *in vitro* cell culture models (Caco-2 or co-culture of Caco-2/HT29-MTX). Nanoemulsions stabilised with lecithin had significantly higher β-carotene in the gastrointestinal digested micellar fraction, lower β-carotene in the Caco-2 (and Caco-2/HT29-MTX) apical compartment and significantly higher β-carotene in Caco-2 cellular content compared to β-carotene-enriched nanoemulsions stabilised with sodium caseinate. Finally, to assess anti-inflammatory activity of digested nanoemulsions, lipopolysaccharide stimulated macrophages were exposed to Caco- 2 basolateral samples with levels of TNF-α and IL-β, subsequently quantified. A TNF-α response from stimulated THP-1 macrophages was elicited by basolateral samples, regardless the emulsifier used to formulate nanoemulsions. This study demonstrated that β-carotene permeability is influenced by the food derived emulsifier used for stabilising nanoemulsions, indicating that composition may be a critical factor for β-carotene delivery.

Keywords: β-carotene, nanoemulsions, *in vitro* digestion, intestinal barrier

1. Introduction

β-carotene is a vitamin A precursor with poor water-solubility (0.0006 g/L at 25ºC). Positive health benefits associated with β-carotene consumption include lower incidence of cancer, cardiovascular diseases and degenerative disorders (Goralcyk, 2009). These health attributes have been related to β-carotene's antioxidant and immunomodulatory bioactivities, proven both *in vitro* (Bai et al., 2005) and *in vivo* (Zhou et al., 2018).

Although the recommended dietary allowance (RDA) for β-carotene has not been set, the U.S. Food and Drug Administration and the European Food Safety Authority derive a RDA for vitamin A of 900-700 μg of retinol activity equivalents (RAE) daily and a population reference intake of 750-650 μg REA daily, respectively. In addition, β-carotene appears to be degraded by the acidic environment of the stomach (Boon, McClements, Weiss, & Decker, 2010) which undoubtedly reduces β-carotene concentration in the intestine.

As a solution, recent studies have revealed the use of nanostructured delivery systems such as nanoemulsions to encapsulate and protect β-carotene after oral consumption and enhance its delivery to intestinal barrier (Chen, Li, Li, McClements, & Xiao, 2017; Gasa-Falcon et al., 2020; Yi, Zhong, Zhang, Yokoyama, & Zhao, 2015). B-carotene enriched nanoemulsions stabilised with pectin, lecithin, sodium caseinate, Tween 20 or sucrose palmitate, have been subjected to *in vitro* gastrointestinal digestion (GID) and the subsequent release of β-carotene has been determined (Gasa-Falcon et al., 2019; Salvia-Trujillo et al., 2013; Teixé-Roig et al., 2020). However, less information is currently available about the use of either proteins or phospholipids-based emulsifiers on β-carotene transit across the intestinal barrier and subsequent bioactivity from nanoemulsions post gastrointestinal digestion (GID). For instance, Lu, Kelly, & Miao (2017) described that the permeability of β-carotene in undifferentiated naïve gastrointestinal epithelial cells is depended on the emulsifier type added to the nanoemulsions rather than initial particle size of the nanoemulsions.

To model absorption *in vitro*, the use of differentiated monolayers expressing tight junctions, best represent the morpho-functional features of the intestinal barrier (Guri, Gülseren, & Corredig, 2013). However, *in vitro* monolayers present their own challenges since digestive fluids and nanoemulsions post GID (micellar fractions) are cytotoxic at relatively low

concentrations (Arranz, Corredig, & Guri, 2016). As a result, quantification of compounds on the basolateral side can be challenging.

Thus, the aim of this study was to evaluate the permeability of β-carotene-enriched nanoemulsions stabilised with two different emulsifiers across 21 days differentiated Caco-2 and Caco-2/HT29-MTX co-cultures, post *in vitro* static GID. These 21 days old Caco-2/HT29-MTX co-cultures best represent the mature intestinal mucus barrier. Emulsifiers (lecithin and sodium caseinate) were selected based on their different properties (low (758 g/mol) and high (≈10-50 KDa) molecular weight), sources (synthetic and natural) and previous physiochemical characterisation of nanoemulsions stabilised with these emulsifiers in our group (Gasa-Falcon et al., 2019). Furthermore, the subsequent basolateral anti-inflammatory activity was assessed by quantification of TNF-α and IL-β in lipopolysaccharide (LPS) stimulated macrophages (THP-1 cells).

2. Materials and methods

2.1. Materials

Corn oil (Mazola, ACH Food Companies Inc., Memphis, TN) was purchased from a local market. β-carotene (synthetic, ≥93% (UV), powder) was sourced from Sigma–Aldrich (Ireland). Lecithin was obtained from Alfa Aesar (Karlsruhe, Germany). Sodium caseinate (NaCas) (≥92% purity) was from Acros Organics (Geel, Belgium). The Caco-2 cell line was purchased from the European Collection of Cell Cultures (ECACC 86010202) and the human monocyte THP-1 (ATCCTIB-202) and the human colon adenocarcinoma HT-29 cell lines (ATCCHTB-38) were purchased from American Type Culture Collection. This latter cell line was differentiated to HT-29-MTX following the protocol described by Guri et al. (2013). Tissue culture plastics were sourced from Sarstedt Ltd. (Wexford, Ireland). CellTiter 96 AQueus One Solution reagent was purchased from Promega (MyBio, Kilkenny, Ireland). Milli-Q water was used to prepare all nanoemulsions. All other chemicals were sourced from Sigma–Aldrich (Ireland) unless specified otherwise.

2.2. Preparation of nanoemulsions

Primary emulsions were prepared by mixing 4% (w/w) of the lipid phase (corn oil enriched with 0.5% w/w of β-carotene) with 96% (w/w) of the aqueous phase containing the emulsifier (lecithin or NaCas) at 2% (w/w). Both phases were mixed with an Ultra-Turrax (IKA, Staufen, Germany) at 9500 rpm for 3 minutes. Then, primary emulsions were passed through an APV 1000 (SPX Flow Technology, Charlotte, NC, USA) at 500 bars for 3 cycles to obtain nanoemulsions.

2.3. Determination of nanoemulsions properties

Particle size of nanoemulsions was determined using a Mastersizer 3000 (Malvern Instruments Ltd, Worcestershire, UK). The results were reported as the surface mean diameter (d_{43} (μm)) and the width of the distribution. The refractive index of the corn oil and water employed to perform the analysis were 1.34 and 1.33, respectively.

The emulsions ζ-potential was determined using a Zetasizer NanoZS (Malvern Instruments Ltd, Worcerstershire, UK). Samples were previously diluted (1/100) and equilibrated prior to analysis.

Physical stability of β-carotene enriched nanoemulsions was determined with an analytical centrifuge LUMiSizer 6112 (L.U.M. GmbH, Berlin, Germany) that accelerates destabilisation of samples. Results were analysed using the software package SEPView 6.0 (L.U.M. GMBH) that records transmitted light across the sample length and calculates the instability index that ranges from 0 to 1, with the greatest instability at 1. Instrumental parameters used for physical stability analysis were: speed 2,186 rcf; time interval 20 seconds; exposure time 10,000 seconds; temperature 25ºC.

2.4. *In vitro* static simulated digestion

Nanoemulsions were subjected to a simulated *in vitro* static GID (gastric and upper intestinal phases) that mimics the adult human upper gut. The INFOGEST standardised method (Brodkorb et al., 2019) was followed with minor modifications. Briefly, gastric phase consisted of 5 mL of nanoemulsion with simulated gastric fluid containing porcine pepsin (EC 3.4.23.1) (3925.3 U/mg); pH was adjusted to 3.0 using HCl (1 M) and volume to 10 mL (Milli-Q water). The mixture was incubated for 2 h at 37°C with continuous shaking in a rotator. After 2h, pH

was increased to 6.5 using NaOH and 20 µL $CaCl_2$ (0.3 M), 4 ml bile (630 g/mol, EC232-369-0), and 2.5 mL pancreatin based on trypsin activity (8.13 U/mg; EC232.468.9) were added. Based on Verkempinck et al. (2017), extra lipase (pancreatin and lipase) was added to reach 420 U/mL. The pH of the mixture was adjusted to 7.0, the volume to 20 mL with milli-Q water, and the mixture was incubated for 2 h at 37°C. The digestion was then stopped by adding protease inhibitor 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (1 mM). To obtain the micellar fraction, the digested fractions were centrifuged (Heraeus Megafuge 1.0, Massachusetts, USA) at 4000 rpm for 40 minutes at 4 °C (Garrett, Failla, Sarama, &Craft, 1999). Samples were stored at −80°C for further experiments.

2.5. *In vitro* cell based assays

Caco-2 and HT29-MTX cell lines were grown in 75 cm^2 tissue culture flasks in a humidified 37 °C incubator with a 5% CO_2 air atmosphere. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 100 U/mL penicillin, and 100 mg/mL streptomycin. At 80% confluency, cells were trypsinated (0.25% trypsin/EDTA), diluted 1:6 in DMEM medium, and reseeded in flasks. Caco-2 and HT29-MTX cell lines in this study were used at passage number 29-41 and 53-67, respectively.

2.6. Cytotoxicity of micellar fractions

Caco-2 cells were seeded at a density of 8×10^4 cells/well in 96-well plate. After 24 h of incubation, cells were washed with PBS. GID micellar fractions were filtrated (0.45 µm), diluted in complete DMEM (between 2-16% v/v) and 80 µL were added to each well. Subsequently, 20 µL of CellTiter 96® AQueous One Solution Cell Proliferation Assay reagent was added to each well and cells were incubated for 2 h. After 2 h, the quantity of formazan produced was measured spectrophotometrically at 490 nm in a microplate reader (Synergy HT BioTek, Winooski, VT, USA). Results were expressed as the percentage of cellular viability relative to a control group (cells with DMEM medium) versus the micellar fraction concentration (%, v/v). Cytotoxicity of pure β-carotene dissolved in DMSO was also evaluated (0.05-10 µg/mL).

2.7. Permeability across intestinal barriers

Caco-2 cells were seeded at a density of 6 × 10^4 cells per insert in 12-well Transwell® plates (0.4 µm pore size, 1.2 cm diameter, Costar, Cambridge, MA). In co-culture experiments, Caco-2 and HT29-MTX were grown separately and then seeded at a ratio of 75:25, to a final density 6 × 10^4 cells per insert.

Culture media of each plate was changed every two days for 21 days. The integrity of the cell monolayer was monitored by measuring the transepithelial electrical resistance (TEER) ($\Omega \cdot cm^2$) using a Millicell-ERS Voltohmmeter (Merck Millipore, Carrigtwohill, County Cork, Ireland). On day 21, apical and basolateral compartments were washed three times with PBS and 470.6 µL and 1500 µL DMEM were added to apical and basolateral compartments, respectively. Then, micellar fractions (29.4 µL) were added to apical compartment and incubated for 2 h. During this 2 h, the TEER value did not change significantly (data not shown).

After permeability experiment, apical and basolateral samples were collected. Moreover, cell monolayer was washed three times with PBS, scraped and collected. Cells were centrifuged (Heraeus Megafuge 1.0) for 3 min at 1100 rpm and the supernatant discarded. Cells were stored at -80 ºC for further analysis.

2.8. Determination of β-carotene

Extraction of β-carotene from samples (apical, cells and basolateral) was performed as described by Yuan, Gao, Zhao, & Mao (2008) with minor modifications. Briefly, the samples were filtrated (0.45 µm) and mixed with ethanol and hexane, followed by a centrifugation (4000 rpm, 5 min, 5ºC) (Heraeus Megafuge 1.0). The upper fraction was collected, dried under N_2 and stored at -80 ºC. Each sample extract was dissolved in 200 µL of the injection solvent acetonitrile (ACN): methanol (MeOH) 7:3 (v/v): acetone 6.7:3.3 (v/v) and filtered through 0.2 µm nylon filters (Millipore, Bedford, MA). High-performance liquid chromatography system (Waters Xevo TQ-S, Milford, USA) equipped with a photodiode array detector (HPLC-PDA) at 450 nm and a column ACQUITY UPLC® (C18 BEH 130 Å, 1.7 µm, 2.1 × 150 mm) (Waters) (30°C and flow rate 0.85 mL/min) were used. Mobile phase consisted of solvent A: ACN: MeOH 7:3 (v/v) and solvent B: water 100%, and the flow was isocratic (100% ACN/MeOH 7/3). β-carotene was

quantified by comparison with external standards. Results were reported as ng/mL and cellular uptake as percentage of detected β-carotene in cells versus apical samples at time 0 h.

2.9. Anti-inflammatory activity of basolateral samples

Human monocytes THP-1 were cultured in RPMI 1640 culture medium supplemented with 10% (v/v) FBS, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified atmosphere. Cells were added at a density of 5 x 10^5 cells/mL in 24 well plates. Differentiation to macrophages was induced by adding 1 µg/µL 12-O-tetradecanoyl phorbol-13-acetate (TPA) to cells followed by 48 h incubation. After differentiation to macrophages, cells were washed with PBS. LPS (0.05 µg/mL) and Caco-2 basolateral samples were added to each well. After 24 h incubation, the culture medium was collected for subsequent TNF-α and IL-1β quantification. Release of TNF-α and IL-1β was measured in the supernatants of THP-1 cells using ELISA kits (R&D Systems, Minneapolis, USA), according to manufacturer's instructions. Multiscanner autoreader (Synergy HT BioTek) was used to read the absorbance of the plates at 450 nm.

2.10. Statistical analysis

Experiments were performed in triplicate on at least two different days and data was expressed as the mean with standard deviation. To determine the statistically significant differences between samples, one-way ANOVA followed by Bonferroni test ($p \leq 0.05$) was conducted with SigmaPlot 11.0.

3. Results and discussion

3.1. Physicochemical properties of nanoemulsions

Both nanoemulsions containing lecithin and NaCas had a monomodal particle size distribution (Fig. 1A) and exhibited particle sizes in the nanometer range (0.35 and 0.29 µm, respectively) (Table 1), in line with our previous published results (Gasa-Falcon et al., 2019). Nanoemulsions had negative ζ-potential values, with lecithin displaying the highest negative value (Table 1).

NaCas-stabilised nanoemulsions exhibited the lowest end point instability index (0.603 ± 0.006) compared with lecithin-stabilised nanoemulsions (0.773 ± 0.001) (Fig. 1B).

Emulsifiers with a low molecular weight and/or with a high hydrophilic–lipophilic balance (HLB) are associated with a high efficiency at producing small particle sizes in oil-in-water emulsions (Jo & Kwon, 2014). Lecithin has an HLB value of 8 while NaCas has a value of 14. Thus, the intermediate-low HLB value of lecithin could explain why nanoemulsions stabilised with this emulsifier exhibited the highest particle size and instability index compared to nanoemulsions with NaCas (Iyer et al., 2015). In addition, the mass of NaCas (≈10-50 KDa) (Ozturk & McClements, 2016), its gelation behaviour (Rodriguez-Patino & Pilosof, 2011) and the thick interfacial layer covering oil droplets (McClements et al., 1993) undoubtedly contributed to its nanoemulsion stability over the accelerated centrifugation process. The large negative ζ-potential values observed for lecithin and NaCas nanoemulsions (-58.81 mV and -53.41 mV, respectively) could be attributed to the phospholipid head groups from lecithin, and the fact that the nanoemulsion pH of ≈6.5 differs to the NaCas isoelectric point (pI=4.6) respectively (Chang & McClements, 2016). Interestingly, ζ-potential of lecithin stabilised β-carotene nanoemulsions became less negative after *in vitro* GID, while NaCas emulsions post GID have a stronger negative value (Gasa-Falcon et al., 2019). In that previous study, particle size after *in vitro* GID in lecithin nanoemulsions was higher compared to NaCas.

3.2. Cytotoxicity of micellar fractions

The maximum non-toxic concentration of micellar fractions was different depending on the emulsifier used (Fig. 2). Micellar fractions with lecithin showed no cell toxicity (>90% cell viability) when Caco-2 cells were exposed to concentrations below 6% (v/v) (Fig. 2A), while for those containing NaCas no cell toxicity was observed at concentrations under 10% (v/v) (Fig. 2B).

In agreement to our results, several studies have demonstrated that emulsifiers are cytotoxic in a concentration-dependent manner and that toxicity of nanoemulsions depends on the nature of emulsifier employed (Buyukozturk, Benneyan, & Carrier, 2010; Ujhelyi et al., 2012). Furthermore, Sadhukha, Layek & Prabha (2018) observed that the aqueous fraction of digested lipid-based delivery systems was responsible for cytotoxicity in MDCK kidney cells, reducing cell

viability by 40%. It has been previously reported that monoglycerides induce dose-dependent apoptosis in mammalian cells (murine thymocytes), which consisted on a rapid reduction in mitochondrial transmembrane potential, production of reactive oxygen species, among other processes (Philippoussos, Arguin, Fortin, Steff, & Hugo, 2002). In our study, micellar factions of nanoemulsions were likely to contain lipid digestion products (i.e. free fatty acids and monoglycerides), and together with emulsifiers contributed to damage the cell integrity.

Similar cell viability results were obtained when control micellar fractions (without β-carotene) were tested in Caco-2 cells, but interestingly control micellar fractions derived from lecithin-stabilised nanoemulsions were 60% less toxic compared to micellar fractions with β-carotene. This suggests that β-carotene plays a role in cell cytotoxicity of Caco-2 monolayers. Indeed, Wooster et al. (2017) observed that the presence of β-carotene in LCT nanoemulsions (IC_{50}= 51 μg/mL) increased four times their toxicity in differentiated Caco-2 cells compared to empty nanoemulsions (IC_{50}= 257 μg/mL). In contrast, a preliminary study by our laboratory indicated that pure β-carotene present in nanoemulsions was not cytotoxic to undifferentiated Caco-2 cells (between 0.05 μg/mL and 10 μg/mL) (data not shown), suggesting that Caco-2 cell monolayers with tight junctions are more sensitive to GID β-carotene-enriched nanoemulsions than undifferentiated Caco-2 cells. Certainly, oxidation products of β-carotene could have been generated during *in vitro* GID, specifically due to the acidic pH of the gastric phase (Failla, Chitchumronchokchai, Ferruzzi, Goltz, & Campbell, 2014). Oxidation of β-carotene can produce carotenoid aldehyde breakdown products, which have documented toxic effects on numerous cell lines (K562, RPE 28 SV4 and ARPE-19) at concentrations between 10–20 μM (Hurst, Saini, Jin, Awasthi, & Van Kuijk, 2005).

To investigate the bioavailability of β-carotene from nanoemulsions, permeability experiments were performed with lecithin and NaCas micellar fractions at a concentration of 6% (v/v).

3.3. Permeability of β-carotene nanoemulsions

After *in vitro* GID, β-carotene concentration present in the micellar fractions was significantly higher in lecithin nanoemulsions compared to those prepared with NaCas (Table 2). There was a significant reduction of β-carotene in apical compartment after 2 h incubation compared to time zero regardless of emulsifier used. Interestingly after 2 h incubation, the apical of NaCas-

283 stabilised nanoemulsions had a significantly higher amount of β-carotene compared to lecithin-
284 stabilised nanoemulsions. β-carotene content in cell lysates of Caco-2 cells was 3 times higher
285 than in Caco-2/HT29-MTX co-cultures. In addition, the β-carotene concentration in Caco-2 cells
286 lysates was significantly higher in lecithin-stabilised nanoemulsions (2.28%) compared with
287 nanoemulsions containing NaCas (1.72%). Concentration of β-carotene was significantly lower
288 in Caco-2/HT29-MTX co-cultures cell lysates (≥0.74%), with no significant differences between
289 both emulsifiers.
290 The reason why NaCas nanoemulsions had less β-carotene in the micellar fraction, the apical
291 sample at time zero and the cell lysate (Caco-2) may be explained by the fact that proteins such
292 as NaCas, can interact hydrophobically with carotenoids and create complexes that act as a
293 physical barrier for gastrointestinal digestive enzymes access (Wackerbarth, Stoll, Gebken,
294 Pelters, & Bindrich, 2009). Hence, β-carotene may have remained entrapped within these
295 complexes and not solubilised within mixed micelles, a process which is essential for
296 permeability across the intestinal barrier (Baskaran, Sugawara, & Nagao, 2003). Moreover,
297 Yang, Decker, Xiao, & McClements (2015) observed that the addition of 36 mg phospholipids
298 (eg. 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine) within the digestive fluids increased the degree
299 of lipid digestion after *in vitro* GID of vitamin E emulsions. This may explain why lecithin-
300 nanoemulsions had higher concentrations of β-carotene within the micellar fraction. Another
301 study observed that the maximum cellular uptake of carotenoids (β-carotene and lutein) in
302 differentiated Caco-2 cells was obtained when micelles contained 50 μmol/L of
303 lysophosphatidylcholine (phospholipid derived from phosphatidylcholine present in lecithin)
304 (Sugawara et al., 2001).
305 In agreement with the present study, Li. Arranz, Guri & Corredig (2017) reported a lower
306 permeability of β-carotene from liposomes using 21-day old Caco-2/HT29-MTX co-cultures
307 compared to Caco-2 monolayers. Interaction with mucus produced by HT29-MTX cell line
308 reduces permeability of mucoadhesive lipophilic molecules, such as β-carotene (Sigurdsson,
309 Kirch, & Lehr, 2013). Co-culturing Caco-2 cells with HT-29MTX adds a further layer of mucus
310 complexity to more closely resemble the *in vivo* environment (Arranz. Corredig & Guri, 2016),
311 but reduces permeability rates which may hamper compound detection. Thus, in our study, the

use of Caco-2/HT29-MTX cell line resulted in lower recovery of β-carotene in the cell lysates regardless of the emulsifier.

The failure to detect β-carotene in basolateral compartment underlined the limitations of the experiment due to upper concentration limits imposed by cytotoxicity data and inadequate sensitivity of detection instrumentation. Also, β-carotene may not have arrived at the basolateral within the 2 h incubation period.

3.4. Immune functionality of basolateral samples

Basolateral samples from permeability assays with β-carotene NaCas nanoemulsions and β-carotene lecithin nanoemulsions significantly increased TNF-α secretion (112% and 124% respectively, $p<0.05$) compared to basolateral sample control (positive control =2291.2±138.5 pg/mL of TNF-α) from LPS activated THP-1 macrophages (Fig. 3A). However, IL-1β levels were unchanged regardless of basolateral samples (4769.4±145.3 pg/mL) (Fig. 3B).

Previous studies have demonstrated that β-carotene reduces levels of TNF-α and IL-1β levels secreted from LPS-stimulated RAW264.7 cells (murine macrophage cell line) and from LPS-treated peritoneal macrophages (Li, Hong, & Zheng, 2019) as well as from serum of BALB/c mice, intraperitoneally injected with β-carotene (10 mg/kg) plus LPS (4 mg/kg) (Bai et al., 2005). This discrepancy with our results may be explained by the bypass of the gut and the use of different test material (β-carotene alone versus basolateral samples of Caco-2 monolayers treated with β-carotene-enriched nanoemulsions). Applying β-carotene directly to LPS stimulated THP-1 cells will dose dependently reduce secreted levels of TNF-α (data not shown). It is important to note that our results do not confirm the presence of β-carotene or metabolites in the basolateral compartment. It is possible that other GID components in the micellar fraction may be capable of modulating TNF-α, although previous studies have shown that lecithin, sodium caseinate or emulsions with different fatty acid composition do not up-regulate cytokine production (Mukhopadhya et al., 2014, Reimund et al., 2004, Treede et al., 2009). However, β-carotene can be metabolised to high molecular weight products (β-apo-8'-carotenal, β-apo-10'-carotenal, β-apo-12'-carotenal, β-apo-14'-carotenal, β-apo-15'-carotenal) and short-chain products (hcyclocitral, β-ionone, ionene, 5,6-epoxi-β-ionone, dihydroactinidiolide and 4-oxo-

ionone) (Siems et al., 2005), which themselves may directly or indirectly act as pro-inflammatory agents (Yeh, Wang, Chen, & Wu, 2009).

4. Conclusions

Bioaccessibility of GID β-carotene in a Caco-2 model was enhanced when β-carotene-enriched nanoemulsions were stabilised with lecithin compared to those stabilised with NaCas. Caco-2 basolateral samples from both nanoemulsions elicited a TNF-α response from stimulated THP-1 macrophages. This study elucidates the importance of nanoemulsion composition for *in vitro* cellular permeability assays and the hurdles faced by concentration limits. Nanostructured food systems using lecithin as emulsifier might be a potential tool to increase uptake of dietary β-carotene.

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References

Arranz, E., Corredig, M., & Guri, A. (2016). Designing food delivery systems: Challenges related to the *in vitro* methods employed to determine the fate of bioactives in the gut. *Food and Function*, *7*, 3319-36.

Bai, S.K., Lee, S.J., Na, H.J., Ha, K.S., Han, J.A., Lee, H., Kwon, Y.G., Chung, C.K., & Kim, Y.M. (2005). β-carotene inhibits inflammatory gene expression in lipopolysaccharide-stimulated

macrophages by suppressing redox-based NF-kB activation. *Experimental & Molecular Medicine, 37*, 323-334.

Baskaran, V., Sugawara, T., & Nagao, A. (2003). Phospholipids affect the intestinal absorption of carotenoids in mice. *Lipids, 38(7)*, 705-711.

Boon, C. S., McClements, D.J., Weiss, J., & Decker E.A. (2010). Factors influencing the chemical stability of carotenoids in foods. *Critical Reviews in Food Science and Nutrition, 50(6)*, 515-32.

Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção R., Balance, S., Bohn, T., Bourlieu-Lacanal, C., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kikhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A.R., Martins, C., Marze, S., McClements, D.J., Ménard, O., Minekus, M., Portmann, R., Santos, C.N., Souchon, I., Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., & Recio, I. (2019). INFOGEST static *in vitro* simulation of gastrointestinal food digestion. *Nature Protocols, 14*(4), 991-1014.

Buyukozturk, F., Benneyan, J.C., & Carrier, R.L. (2010). Impact of emulsion-based drug delivery systems on intestinal permeability and drug release kinetics. *Journal of Controlled Release, 142* (1), 22-30.

Chang, Y., & McClements, D.J. (2016). Influence of emulsifier type on the in vitro digestion of fish oil-in-water emulsions in the presence of an anionic marine polysaccharide (fucoidan): Caseinate, whey protein, lecithin or Tween 80. *Food Hydrocolloids, 61*, 92-101.

Chen, J., Li, F., Li, Z., McClements, D.J., & Xiao, H. (2017). Encapsulation of carotenoids in emulsion-based delivery systems: Enhancement of β-carotene water dispersibility and chemical stability. *Food Hydrocolloids, 69*, 49-55.

~~Elmadfa, I., Weichselbaum, E., Koenig, J., Remau de Winter, A.M., Trolle, E., Haapala, I., Uusitalo, U., Mennen, L., Hercberg, S., Wolfram, G., Trichopoulou, A., Naska, A., Benetou, V., Kritsellis, E., Rodler, I., Zajkás, G., Branca, F., D'Acapito, P., Klepp, K.I., Ali-Madar, A., Vaz de Almeida, M.D., Alves, E., Rodrigues, S., Serra-Majem, L., Roman, B., Sjostrom, M., Poortvliet, E., & Margetts, B.(2005). European Nutrition and Health report 2004. *Animals of Nutrition and Metabolism, 48*(2), 1-2.~~

398 Failla, M.L., Chitchumronchokchai, C., Ferruzzi, M.G., Goltz, S.R., & Campbell, W.W. (2014).
399 Unsaturated fatty acids promote bioaccessibility and basolateral secretion of carotenoids and α-
400 tocopherol by Caco-2 cells. *Food and Function, 5*(6), 1101-1112

401 Garrett, D.A., Failla, M.L., Sarama, R.J., & Craft, N. (1999). Accumulation and retention of
402 micellar β-carotene and lutein by Caco-2 human intestinal cells. *Journal of Nutritional*
403 *Biochemistry, 10*(10), 573-581.

404 Gasa-Falcon, A., Odriozola-Serrano, I., Oms-Oliu, G., Martín-Belloso, O. (2019). Impact of
405 emulsifier nature and concentration on the stability of β-carotene enriched nanoemulsions
406 during in vitro digestion*. Food and Function, 10,* 713-722.

407 Gasa-Falcon, A., Odriozola-Serrano, I., Oms-Oliu, G., Martín-Belloso, O. (2020).
408 Nanostructured lipid-based delivery systems as a strategy to increase functionality of bioactive
409 compounds. *Foods, 9*(3), 325.

410 Goralcyk, R. (2009). β-carotene, and lung cancer in smokers: review of hypotheses and status
411 of research. *Nutrition and Cancer, 61* (6), 767-774.

412 Guri, A., Gülseren, I., & Corredig, M. (2013). Utilization of solid lipid nanoparticles for enhanced
413 delivery of curcumin in cocultures of HT29-MTX and Caco-2 cells*. Food and Function, 4*(9),
414 1410-1419.

415 Hurst, J.S., Saini, M.K., Jin, G.F., Awasthi, Y.C., & Van Kuijk, F.J.G.M. (2005). Toxicity of
416 oxidized β-carotene to cultured human cells. *Experimental Eye Research, 81*(2), 239-243.

417 Iyer, V., Cayatte, C., Guzman, B., Schneider-Ohrum, K., Matuszak, R., Snell, A., Manohar-
418 Rajani, G., McCarthy, M.P., & Muralidhara, B. (2015). *Human Vaccines and*
419 *Immunotherapeutics, 11*(7), 1853-1864.

420 Jo, Y., & Kwon, Y.J. (2014). Characterization of β-carotene nanoemulsions prepared by
421 microfluidization technique. *Food Science and Biotechnology, 23*(1), 107-113.

422 Li, R., Hong, P., & Zheng, X. (2019). β-carotene attenuates lipopolysaccharide-induced
423 inflammation via inhibition of the NF-κB, JAK2/STAT3 and JNK/p38 MAPK signaling pathways
424 in macrophages*. Animal Science Journal, 90*(1), 140-148.

425 Li, Y., Arranz, E., Guri, A., &Corredig, M. (2017). Mucus interactions with liposomes
426 encapsulating bioactives: Interfacial tensiometry and cellular uptake on Caco-2 and co-cultures
427 of Caco-2/HT29-MTX. *Food Research International, 92*, 128-137.

428 Lu, W., Kelly, A., & Miao, S. (2017). Bioaccessibility and cellular uptake of β-carotene
429 encapsulated in model o/w emulsions: influence of initial droplet size and emulsifiers.
430 *Nanomaterials, 7*(9), 282.

431 McClements, D.J., Dickinson, E., Dungan, S.R., Kinsella, J.E., Ma, J.G., & Povey, M. J. W.
432 (1993). Effect of emulsifier type on the crystallization kinetics of oil-in-water emulsions
433 containing a mixture of solid and liquid droplets. *Journal of Colloids and Interface Science,*
434 *160*(2), 293-297.

435 Mukhopadhya, A., Noronha, N., Bahar, B., Ryan, M.T., Murray, B.A., Kelly, P.M., O'Loughlin,
436 I.B., O'Doherty, J.V., & Sweeney, T. (2014). *Food Science and Nutrition, 2*(6), 712-723.

437 Ozturk, B., & McClements, D.J. (2016). Progress in natural emulsifiers for utilization in food
438 emulsions. *Current Opinion in Food Science, 7*, 1-6.

439 Philippoussis, F., Arguin, C., Fortin, M., Steff, A.M., & Hugo, P. (2002). Cellular specificity
440 related to monoglyceride-induced cell death. *Immunology Letters, 83*(3), 221-230.

441 Reimund, J.M., Scheer, O., Muller, C.D., Pinna, G., Duclos, B., & Baumann, R. (2004). *In vitro*
442 modulation of inflammatory cytokine production by three lipid emulsions with different fatty acid
443 compositions*. Clinical Nutrition, 23*(6), 1324-1332.

444 Rodriguez-Patino, J.M., & Pilosof, A.M.R. (2011). Protein-polysaccharide interactions at fluid
445 interfaces*. Food Hydrocolloids, 25*(8), 1925-1937.

446 Sadhuka, T., Layek, B., & Prabha, S. (2018). Incorporation of lipolysis in monolayer permeability
447 studies of lipid-based oral drug delivery systems. *Drug Delivery and Translational Research,*
448 *8*(2), 375-386.

449 Salvia-Trujillo, L., Qian, C., Martín-Belloso, O., McClements, D.J. (2013). Influence of particle
450 size on lipid digestion and β-carotene bioaccessibility in emulsions and nanoemulsions. *Food*
451 *Chemistry, 141*(2), 1472-80.

452 Siems, W., Wiswedel, I., Salermo, C., Crifò, C., Augustin, W., Schild, L., Langhans, C.D., &
453 Sommerburg, O. (2005). β-carotene breakdown products may impair mitochondrial functions-
454 Potential side effects of high-dose β-carotene supplementation. *Journal of Nutritional*
455 *Biochemistry, 16*(7), 358-397.

456 Sigurdsson, H.H., Kirch, J., & Lehr, C.M. (2013). Mucus as a barrier to lipophilic drugs.
457 *International Journal of Pharmaceutics, 453*(1), 56-64.

458 Sugawara, T., Kushiro, M., Zhang, H., Nara, E., Ono, H., & Nagao, A. (2001).
459 Lysophosphatidylcholine enhances carotenoid uptake from mixed micelles by Caco-2 human
460 intestinal cells. *The Journal Nutrition, 131*(11), 2921-2927.

461 Teixé-Roig, J., Oms-Oliu, G., Ballesté-Muñoz, S., Odriozola-Serrano, I., Martín-Belloso, O.
462 Improving the *in vitro* bioaccessibility of β-carotene using pectin added nanoemulsions. *Foods,*
463 *9*(4), 447.

464 Treede, I., Braun, A., Jeliaskova, P., Giese, T., Füllekrug, J., Griffiths, G., Stremmel, W., &
465 Ehehalt, R. (2009). TNF-alpha-induced up regulation of pro-inflammatory cytokines is reduced
466 by phosphatidylcholine in intestinal epithelial cells. *BMC Gastroenterology, 13*, 9-53.

467 Ujhelyi, Z., Fenyvesi, F., Váradi, J., Fehér, P., Kiss, T., Veszelka, S., Deli, M., Vecsernyés, M., &
468 Bácskay, I. (2012). Evaluation of cytotoxicity of surfactants used in self-micro emulsifying drug
469 delivery systems and their effects on paracellular transport in Caco-2 cell monolayer. *European*
470 *Journal of Pharmaceutical Sciences, 47*(3), 564-573.

471 Verkempinck, S.H.E., Salvia-Trujillo, L., Moens, L.G., Charleer,L., Van Loey, A.M., Hendrickx,
472 M.E., & Grauwet, T. (2017). Emulsion stability during gastrointestinal conditions effects lipid
473 digestion kinetics. *Food Chemistry, 246*, 179-191.

474 Wackerbarth, H., Stoll, T., Gebken, S., Pelters, C., & Bindrich, U. (2009). Carotenoid-protein
475 interaction as an approach for the formulation of functional food emulsions. *Food Research*
476 *International, 42*(9), 1254-1258.

477 Wooster, T.J., Moore, S.C., Chen, W., Andrews, H., Addepalli, R., Seymour, R.B., & Osborne,
478 S.A. (2017). Biological fate of food nanoemulsions and the nutrients they carry-internalisation,

transport and cytotoxicity of edible nanoemulsions in Caco-2 intestinal cells. *RSC Advances, 7*(64), 40053-40066.

Yang, Y., Decker, E.A., Xiao, H., & McClements, D.J. (2015). Enhancing vitamin E bioaccessibility factors impacting solubilization and hydrolysis of α-tocopherol acetate encapsulated in emulsion-based delivery systems. *Food & Function, 6*(1), 84-97.

Yeh, S.L., Wang, H.M., Chen, P.Y., & Wu, T.C. (2009). Interactions of β-carotene and flavonoids on the secretion of pro-inflammatory mediators in an in vitro system. *Chemico-Biological Interactions, 179*(2-3), 386-393.

Yi, J., Zhong, F., Zhang, Y., Yokoyama, W., & Zhao, L. (2015). Effects of lipids on *in vitro* release and cellular uptake of β-carotene in nanoemulsion-based delivery systems. *Journal of Agricultural and Food Chemistry, 63*(50), 10831-10837.

Yuan, Y., Gao, Y., Zhao, J. & Mao, L. (2008). Characterization and stability evaluation of β-carotene nanoemulsions prepared by high pressure homogenization under various emulsifying conditions. *Food Research International, 41*(1), 61-68.

Zhou, L., Ouyang, L., Lin, S., Chen, S., Liu, Y.J., Zhou, W., & Wang, X. (2018). Protective role of β-carotene against oxidative stress and neuroinflammation in a rat model of spinal cord injury. *International Immunopharmacology, 61*, 92-99.

Table and figures captions

Table 1. Particle size (μm) and ζ-potential (mV) of nanoemulsions stabilised with different emulsifiers (LE: lecithin; NaCas: sodium caseinate). Differences among nanoemulsions were compared using one-way ANOVA followed by Bonferroni test. Different letters indicate statistically significant differences within the parameter tested (p<0.05).

Table 2. β-carotene concentration (ng/mL) quantified by high-performance liquid chromatography (HPLC-PDA) in micellar fractions (after *in vitro* digestion), apical samples and basolateral samples from permeability experiments after 2h incubation with micellar fractions obtained after *in vitro* digestion of nanoemulsions stabilised with lecithin (LE) and sodium caseinate (NaCas). ND = not detected, i.e., below detection limit of 10 ng/ml. Within a row, different lowercase letters indicate statistically significant differences (p<0.05) between emulsifiers. For an emulsifier, a statistical difference between apical t=0 and apical t=2h is denoted by *. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*p<0.05).

Fig 1. (A) Particle size distribution and (B) instability profile of β-carotene-enriched nanoemulsions (0.02% β-carotene w/w, 4% corn oil w/w) stabilised with 2% of lecithin (LE) or NaCas: sodium caseinate (NaCas).

Fig 2. Cell viability (%) of Caco-2 cells after 2h incubation with micellar fractions (with β-carotene) (% v/v) and control micellar fractions (without β-carotene) (% v/v) obtained after *in vitro* digestion of nanoemulsions stabilised with different emulsifiers. Micellar fractions containing lecithin (A) and NaCas (B) were diluted with complete DMEM. Control cells (Ctrl) were grown in media with no treatment (100% viability). Different uppercase and lowercase letters indicate significant differences to control cells for micellar fractions and control micellar fractions, respectively. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*p<0.05). Percentage of cell viability above 80% was considered as non-cytotoxic.

Fig 3. Effects of basolateral samples resulted from permeability experiments with β-carotene-enriched nanoemulsions emulsified with either 2% sodium caseinate (NaCas+) or lecithin (LE+) on the secretion (% ± SEM) of TNF-α (A) and IL-1β (B) in lipopolysaccharide (LPS)-simulated THP-1 cells. Positive controls (Ctrl +) were LPS-stimulated THP-1 cells and negative controls (Ctrl -) were non-stimulated THP-1 cells. Both controls were incubated with basolateral samples collected from control Caco-2 monolayers. * Indicates significant differences to positive controls. Statistical analysis was performed using one-way ANOVA followed by Bonferroni test (*p<0.05).

1 **Table 1.**

Emulsifier	Particle size (μm)	ζ-potential (mV)
LE	0.35 ± 0.001[a]	-58.81 ± 2.56[a]
NaCas	0.29 ± 0.001[b]	-53.41 ± 1.83[b]

2

3

4

Table 2

Table 2.

	Caco-2 model (ng/mL)		Caco-2/HT29-MTX model (ng/mL)	
Emulsifier	**LE**	**NaCas**	**LE**	**NaCas**
Micellar fraction	36430.29 ± 664.67 [a]	32858.26 ± 70.23 [b]	37370.27 ± 521.04 [a]	32758.94 ± 102.51 [b]
Apical t=0h	2170.60 ± 39.09 [a*]	1929.91 ± 4.13 [b*]	2198.25 ± 40.13 [a*]	1926.99 ± 6.04 [b*]
Apical t=2h	251.30 ± 13.99 [a]	338.54 ± 64.99 [b]	263.29 ± 35.64 [a]	364.75 ± 43.67 [b]
Cells	49.56 ± 5.66 [a]	33.19 ± 8.37 [b]	15.14 ± 5.75 [c]	18.12 ± 11.19 [c]
Basolateral	ND	ND	ND	ND

Figure 1

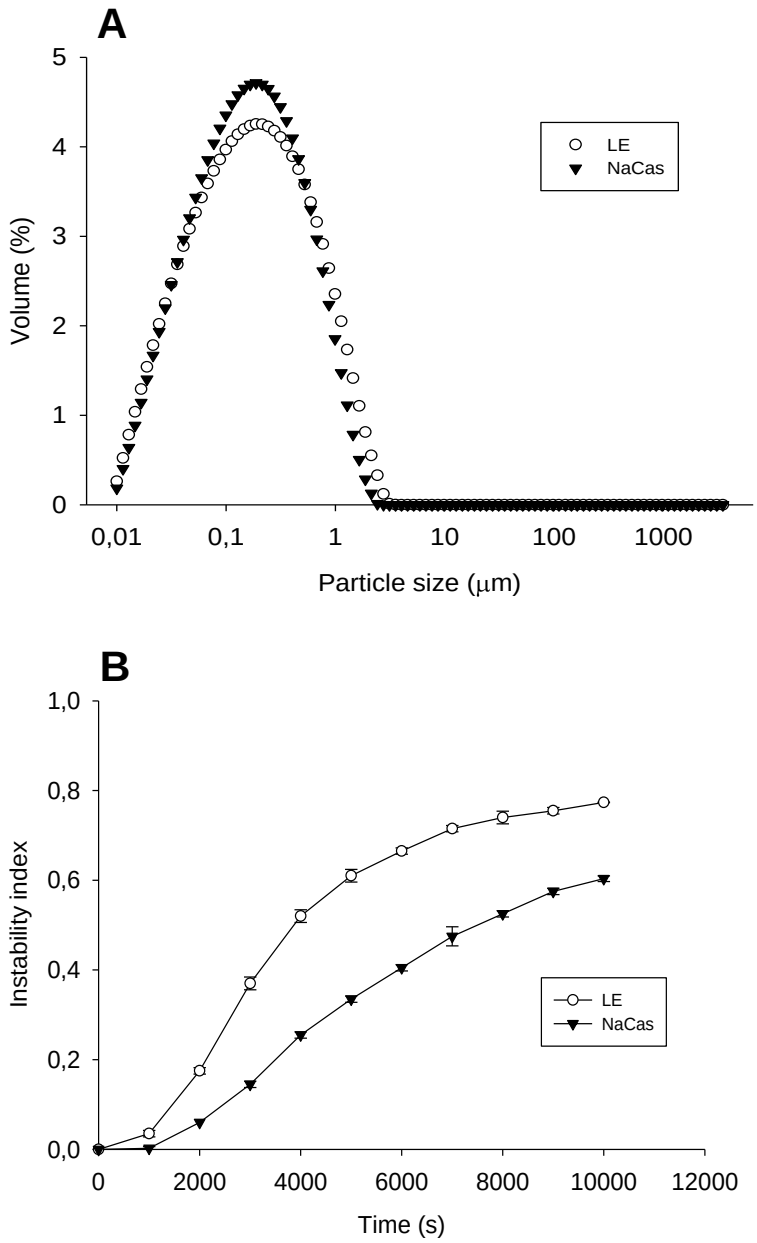

A
Volume (%)
Particle size (μm)
LE
NaCas
B
Instability index
Time (s)
LE
NaCas

Figure 2

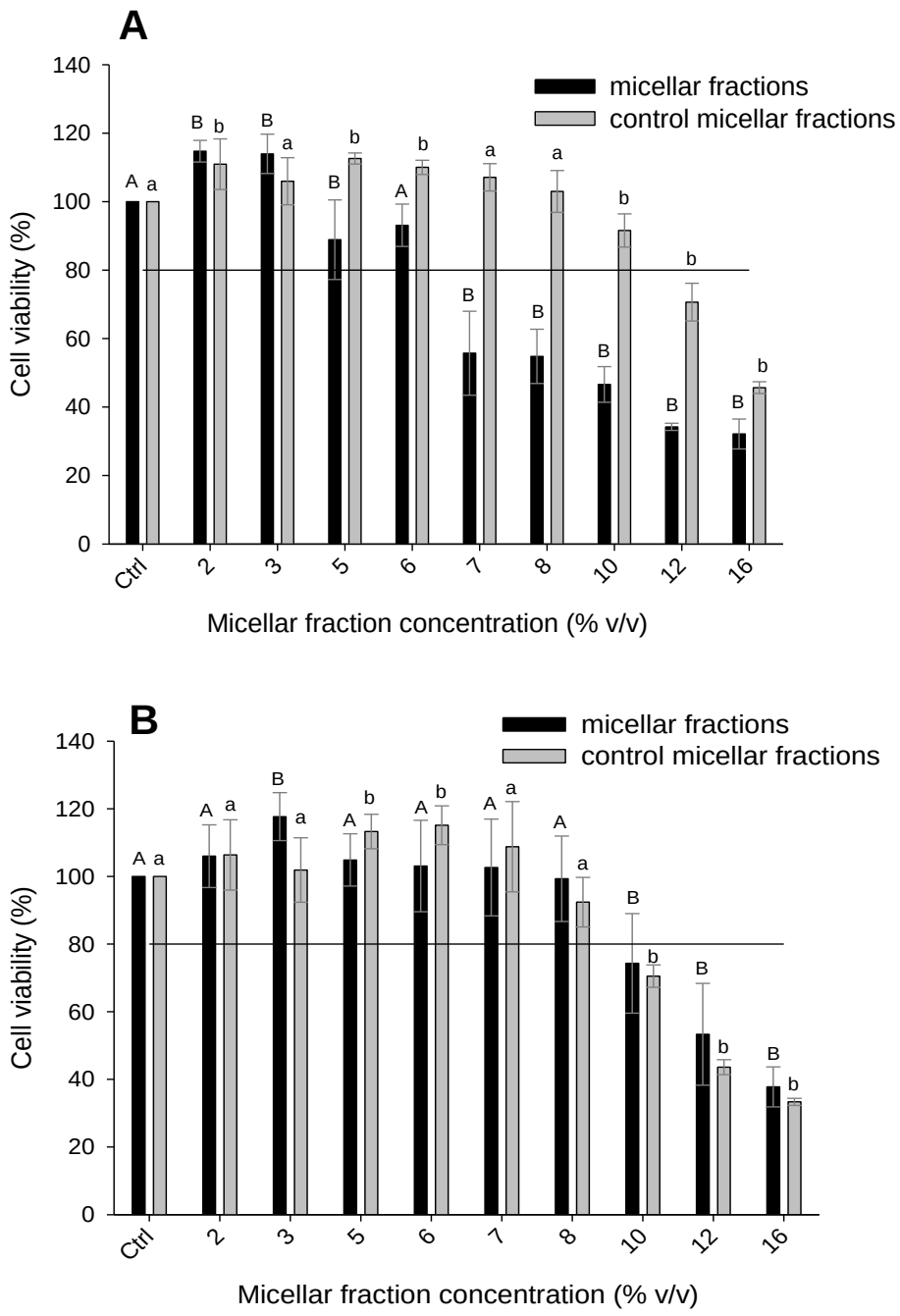
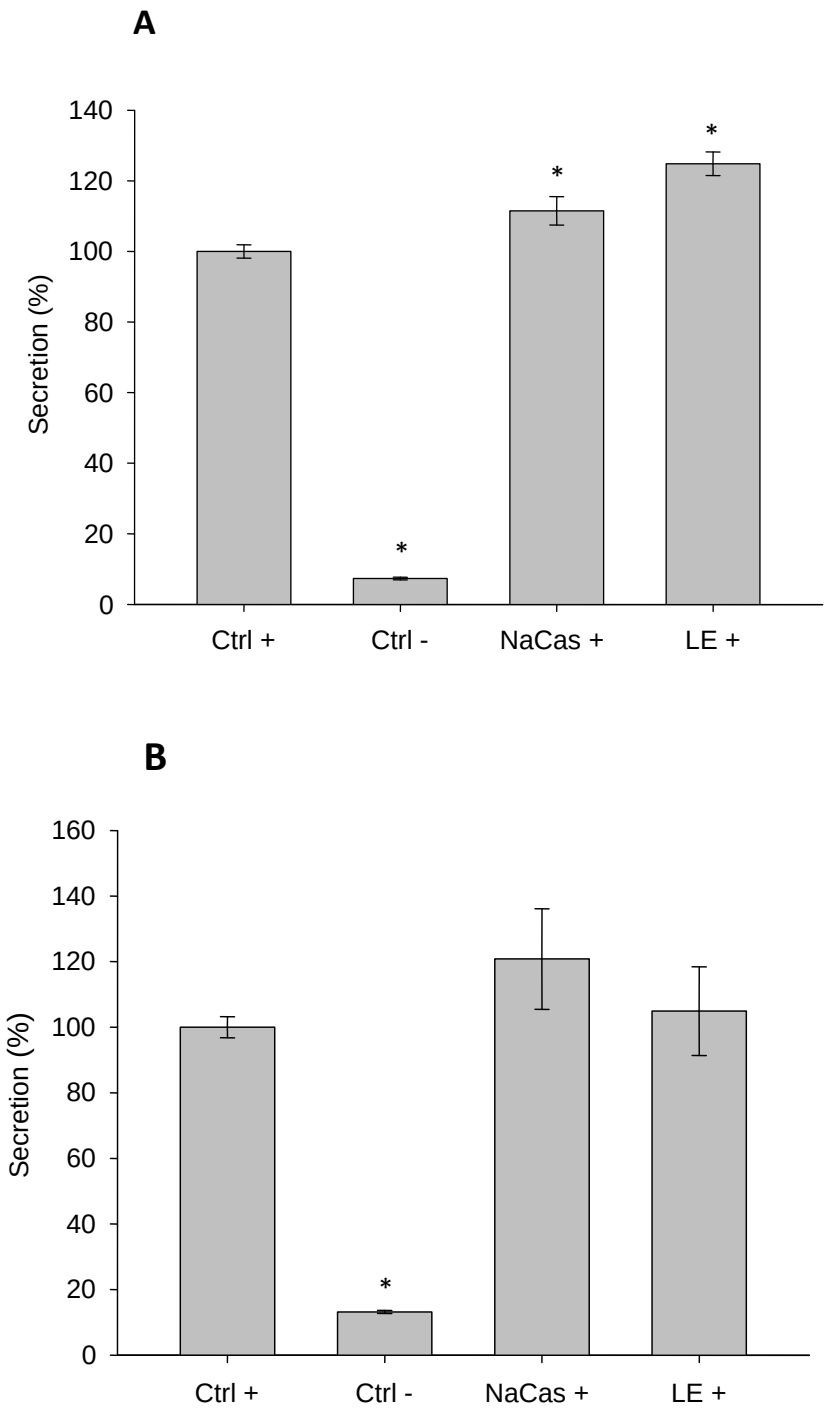

A
B
micellar fractions
control micellar fractions
Cell viability (%)
Micellar fraction concentration (% v/v)
Ctrl
2
3
5
6
7
8
10
12
16
0
20
40
60
80
100
120
140

Figure 3


A
Secretion (%)
140
120
100
80
60
40
20
0
*
*
*
Ctrl +
Ctrl -
NaCas +
LE +
B
Secretion (%)
160
140
120
100
80
60
40
20
0
*
Ctrl +
Ctrl -
NaCas +
LE +

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***Conflict of Interest Form**

The authors declare that there are no conflicts of interest.